Partenariat Analyse Authenticité Arômes

P3A

Focus
The role of chiral analysis in checking the regulatory compliance of natural flavourings

October 2022

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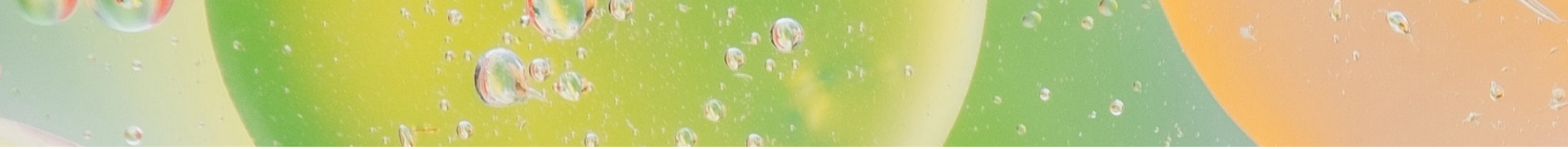


This document has been prepared by the Scientific Committee of the Partenariat Analyse Authenticité Arômes (Flavouring Authenticity Analysis Partnership or P3A). P3A was set up by SNIAA (French Flavour Association) in conjunction with several analytical laboratories with the aim of boosting confidence both in the results produced by analytical laboratories and in the products offered by flavouring manufacturers.

Members of the P3A are indeed frequently called upon to conduct analyses to confirm the compliance of natural flavourings.

The P3A Scientific Committee has decided to draft a series of documents to provide practical and explanatory information regarding the role of each of these analytical methods available for checking the regulatory compliance of natural flavourings.

1.Definition of chirality

Chirality is a concept linked to three-dimensional geometry in space and has an important application in its ability to describe the stereochemistry of molecules forming three-dimensional structures. Stereochemistry describes the relative spatial arrangement of atoms forming the structure of molecules. A molecule is called **chiral** if it cannot be superposed on its mirror image. Comparable examples of chirality in everyday life are snail shells and corkscrews.

Molecular asymmetry results from the presence of stereogenic centres within molecules. There are several categories. **Asymmetric carbons** are the stereogenic centres most commonly found in organic molecules. An asymmetric carbon is, by definition, a **sp3 hybridised carbon atom bonded to four different types or groups of atoms**.[1] The resulting asymmetry within the molecule will induce different physico-chemical properties, depending on the enantiomer.

When a molecule is chiral, it has two forms known as **enantiomers** or **optical isomers**. This name derives from a fundamental property of chiral molecules: the fact that they are active in polarised light. An enantiomer that deflects the plane of polarised light 'to the left' (at a negative angle; anti-clockwise) is referred to as **laevorotatory** (from the Latin laevus for left). This is denoted **l- or (-)**, whereas the **dextrorotatory** form, which deflects the plane of polarised light 'to the right' (from the Latin dexter for right), is denoted **d- or (+)**[2] (figure 1).

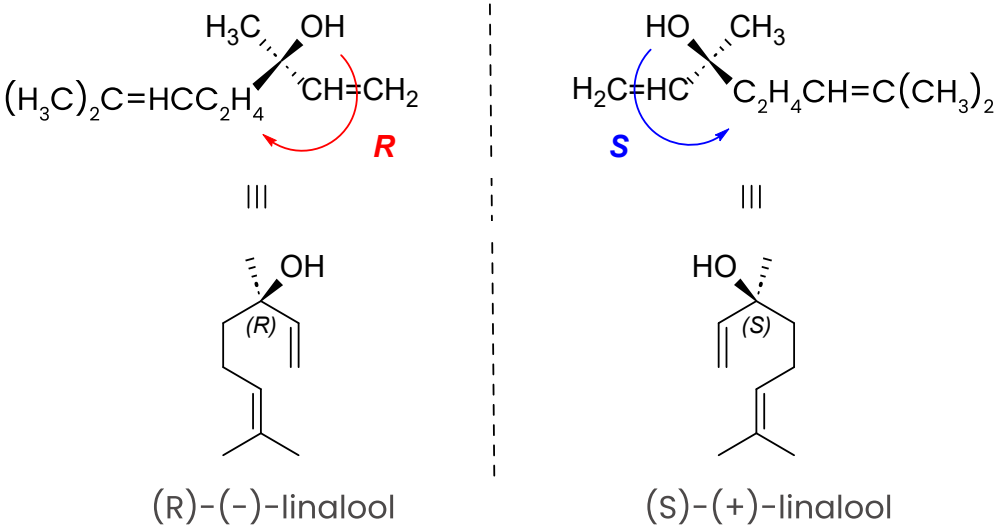


Figure 1. (S)-(+) and (R)-(-) enantiomers of linalool (molecular formula $C_{10}H_{18}O$)

[1] According to the Cahn Ingold Prelog sequence rules, or CIP priority rules, it is possible to determine the absolute configuration - descriptor R or S - of an asymmetric carbon atom. Appropriate definitions can be found in any undergraduate organic chemistry textbook. In addition to the usual descriptors of chirality (+) or (-), l- or d-, a chiral molecule can also be described using its absolute configuration descriptors, such as **(R)-(+)-limonene**, which is found in abundance in citrus essences.

[2] The descriptors l- and d-, which describe the optical activity of the molecule (laevorotatory or dextrorotatory form), should not be confused with the descriptors L- and D-, which relate only to the determination of the Fischer configuration (e.g. L-alanine, D-glucose, etc.).

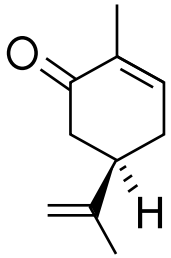
The combination of both enantiomers in equal proportions is referred to as a **racemic mixture**. When the quantity of one enantiomer exceeds the other, the term **enantiomeric excess (ee)** is usually adopted to describe the proportion between the two forms. By way of illustration, this example takes two enantiomers denoted (-)-E_L and (+)-E_D, present in the respective proportions of 96:4. The enantiomeric excess is calculated by applying the following relationship:

$$ee = 100 \times \frac{(E_L - E_D)}{(E_L + E_D)}$$

It can therefore be determined that on the basis of the proportions indicated, the (-)-EL_L enantiomer exhibits an enantiomeric excess of 92%.

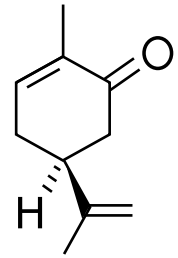
The majority of organic molecules found in **natural extracts** are all **derived from metabolic processes** that involve a battery of enzymes in catalysing the reactions that result in the formation of basic structures. Since enzymes are themselves chiral structures (formed from amino acids), these primary metabolic reactions are often accompanied by establishment of the **absolute configuration of key asymmetric carbons**, which will subsequently determine the configuration of other asymmetric carbons, particularly when the various skeletons are functionalised. All of which often results in structural complexity, which therefore originates in the biochemical arsenal of the plant, and therefore its genetic heritage. So for certain common chiral molecules, such as certain terpenoids, esters and lactones, **their chirality is a function of their original matrix**, and for some molecules, we can even go as far as to determine **enantiomeric excesses characteristic** of the extracts in which they are present.

Lastly, we cannot conclude on this subject without mentioning **the obvious link between the chirality of a molecule and its organoleptic character**. The odour of an organic molecule is determined by a number of physico-chemical factors (volatility, polarity, etc.), but in this case, the main parameter is its **ability to activate one or more types of neuronal receptor** located in the olfactory epithelium of the nasal cavity. Like enzymes, these receptors are functional proteins composed of amino acids. Olfactory receptors are therefore chiral structures that will have a greater or lesser degree of affinity, depending on the enantiomer of the odorant molecule in question. It is not uncommon for one of the two enantiomers to be odorous, while the other is much less potent, has an unpleasant odour, or is even completely odourless. This is referred to as chiral recognition (figure 2).



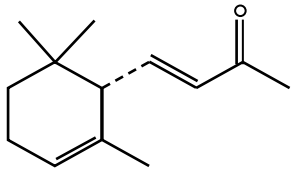
(R)

(R)-(-)-carvone: spearmint odour

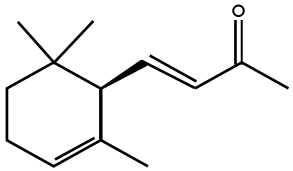


(S)

(S)-(+)-carvone: caraway odour



(S)-(-)-alpha ionone: woody, cedarwood-type odour



(R)-(+)-alpha ionone: violet odour, fruity type, raspberry

Figure 2. Examples of enantiomers with different organoleptic properties

Determining the chirality of organic molecules in natural extracts has always been a major issue in many areas of chemistry, from flavour and fragrance chemistry to pharmaceuticals. For more than seventy years and following the advent of modern chromatographic techniques (GC and LC), a great deal of intensive research has been focused on this subject at international level with the stated aim of tracing the naturalness of natural extracts by examining the chirality of their constituents.

The purpose of this document is to

- 1) **list and describe the methodologies used to analyse chirality** in natural extracts, with particular emphasis on flavouring molecules,

- 2) lay the foundations for **building a database** containing the majority of literature on this subject, and

- 3) examine how the determination of **chirality in natural extracts impacts their regulatory compliance.**

2.Determination of chirality - Enantioselective analysis

2.1. Chiral selectors

In the world of flavourings and fragrances, it is commonplace to hear - inaccurately - that a particular molecule has been analysed using *'Chiral GC (or LC)'*. Strictly speaking, a gas (or liquid) chromatograph is not chiral, and neither is the separation column used in this analytical technique. On the other hand, the stationary phases used are formulated on the basis of a conventional achiral substrate (PDMS, OV1701, etc.) in which a **chiral selector** has been dissolved/suspended to obtain variable chiral recognition with the molecules under analysis.

So for a given chiral molecule, if one of the two enantiomers shows greater affinity than the other towards the chosen chiral selector, the result is higher chromatographic retention for that enantiomer. The outcome is therefore **enantioselective separation**.

The chiral selectors most commonly used in chromatography are derived either from amino acids or sugars (cyclodextrins,amylose, cellulose, etc.). The **cyclodextrins** used in the majority of columns to analyse volatile compounds are **cyclic oligomers of glucose** whose overall shape is often likened to that of a truncated cone. The size of the cone also varies with the number of glucose units constituting the cyclodextrin. The 3 main types are alpha (6 glucose units), beta (7 units) and gamma (8 units) cyclodextrins (figure 3).

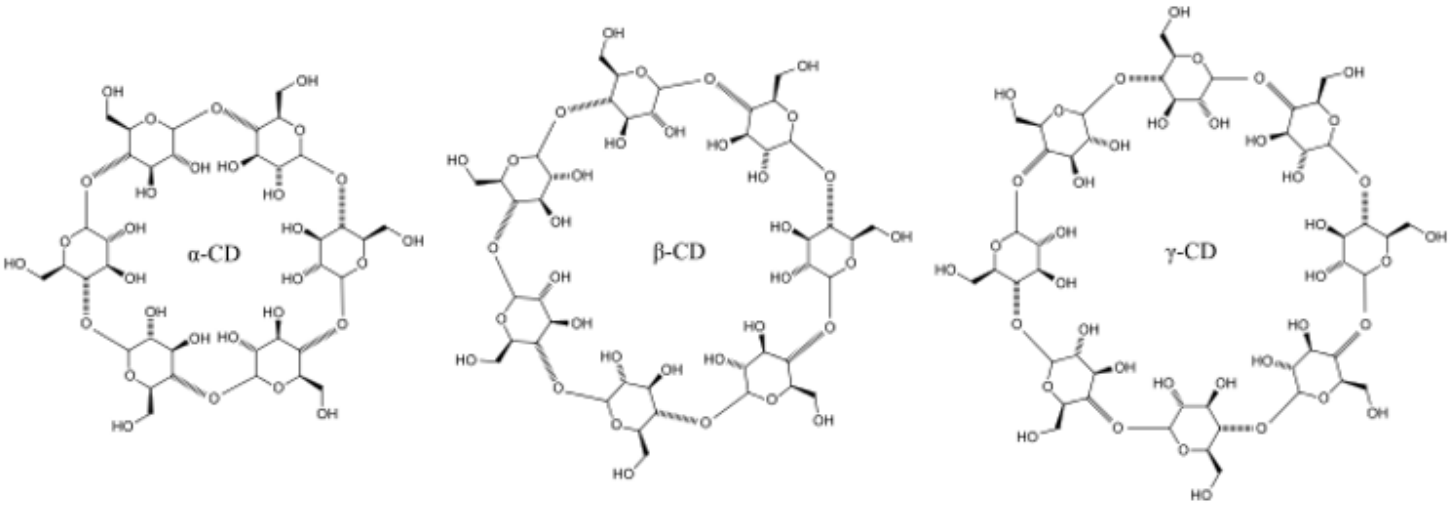


Figure 3. The basic structures of the most common cyclodextrins

In chromatography, and particularly in the context of determining the chirality of volatile compounds, beta- and gamma-cyclodextrins are those that have resulted in the most commonly used analytical applications. These are variously grafted in positions 2, 3 and 6 of the glucose units (figure 4) by organic groups, such as:

- ethers (O-methyl, O-pentyl, O-methoxymethyl, etc.),
- esters (O-Ac, O-propionyl, O-butyryl, O-TFA, etc.),
- silylated groups (O-TMS, O-TBDMS, O-THS, etc.) found almost exclusively in position 6.

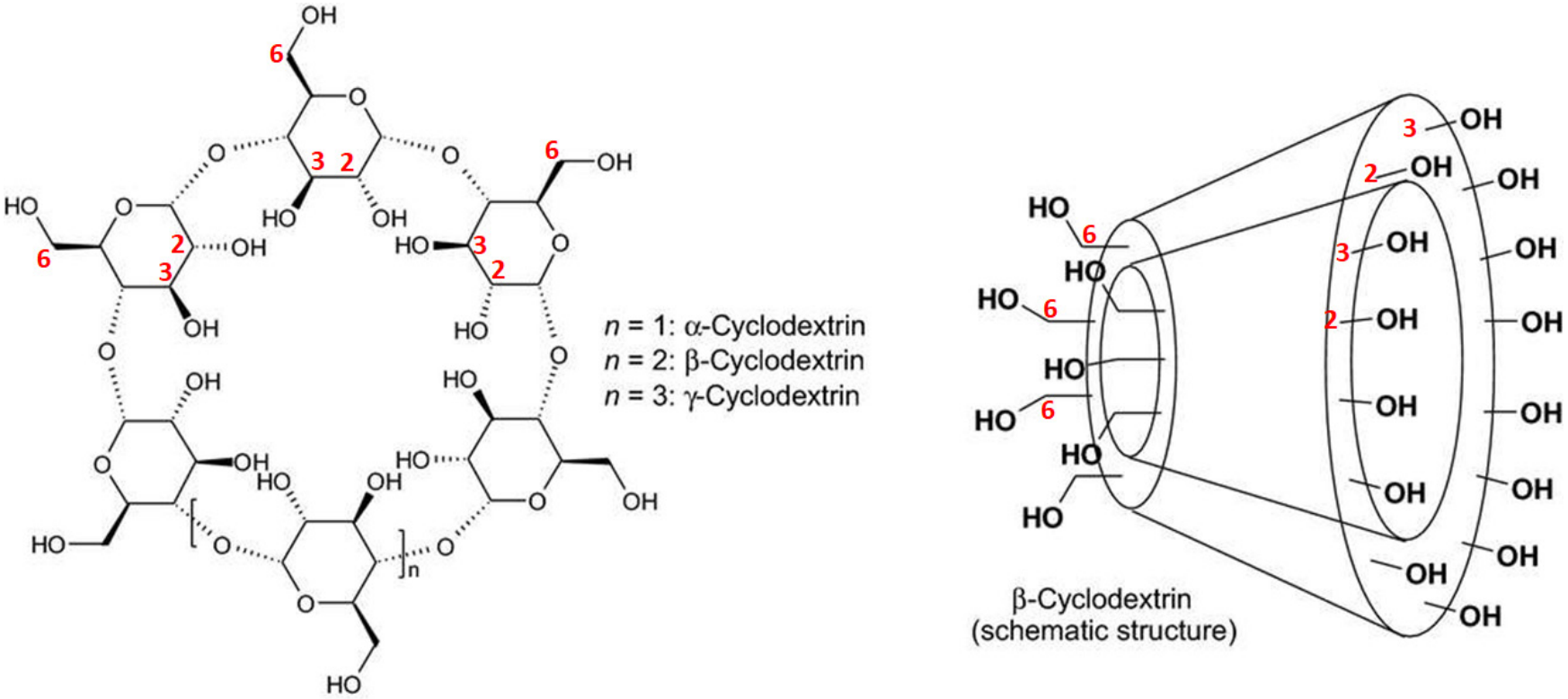


Figure 4. Three-dimensional representation of a cyclodextrin

As a result, there is a wide range of so-called 'chiral' columns available on the market and prepared from ß- or γ-cyclodextrins (more rarely α). These are variously substituted in the positions referred to above and are recommended either by manufacturers or the relevant scientific literature, depending on the target to be analysed.

2.2. Experimental implementation

Despite many attempts to rationalise the host-ligand recognition processes that exist between cyclodextrins and their target analytes (lactones, terpenes, etc), it is not currently possible to predict that a well-defined cyclodextrin will be particularly suitable for separating the enantiomers of a particular molecule.

Enantiomer separation involves a range of different chiral recognition phenomena that are not limited to the simple process of including the host in the hydrophobic cavity of the cyclodextrin. It has been demonstrated that chiral recognition can also occur outside the cone. This is why **analysts working on authenticity and naturalness will be obliged to screen as many 'chiral' columns as possible** in order to determine which stationary phase is best suited to the separation they wish to achieve. A number of experimental parameters need to be closely controlled to maximise the chances of separation (figure 5):

- Firstly, the analyst must have access to the **racemic mixture** required to develop the right analytical conditions, or at least a mixture in which the 2 enantiomers are present in clearly visible quantities, in order that they can assess the **degree of peak separation by resolution measurement.**

- To determine the **order of elution** of the enantiomers, at least one **defined standard of chirality** (in the best case, for both enantiomers) will be required in addition to the racemic mixture.

- It may be helpful to use a **mass spectrometer** for detection in order to confirm peak identification and correct separation of the **2 enantiomers** (there must be **2 distinct peaks with the same mass spectrum**).

Although in principle any type of chromatography (liquid, supercritical fluid, gas, etc.) can be used to separate the enantiomers of a compound, in practice, **gas chromatography is generally preferred** due to the volatile nature of flavouring compounds. A **universal FID-type detector can be used, but mass spectrometry is the preferred option**, because the identity and purity of the eluted peaks can be confirmed by comparing the spectra with those of authentic reference compounds analysed under the same conditions, or by searching mass spectral libraries.

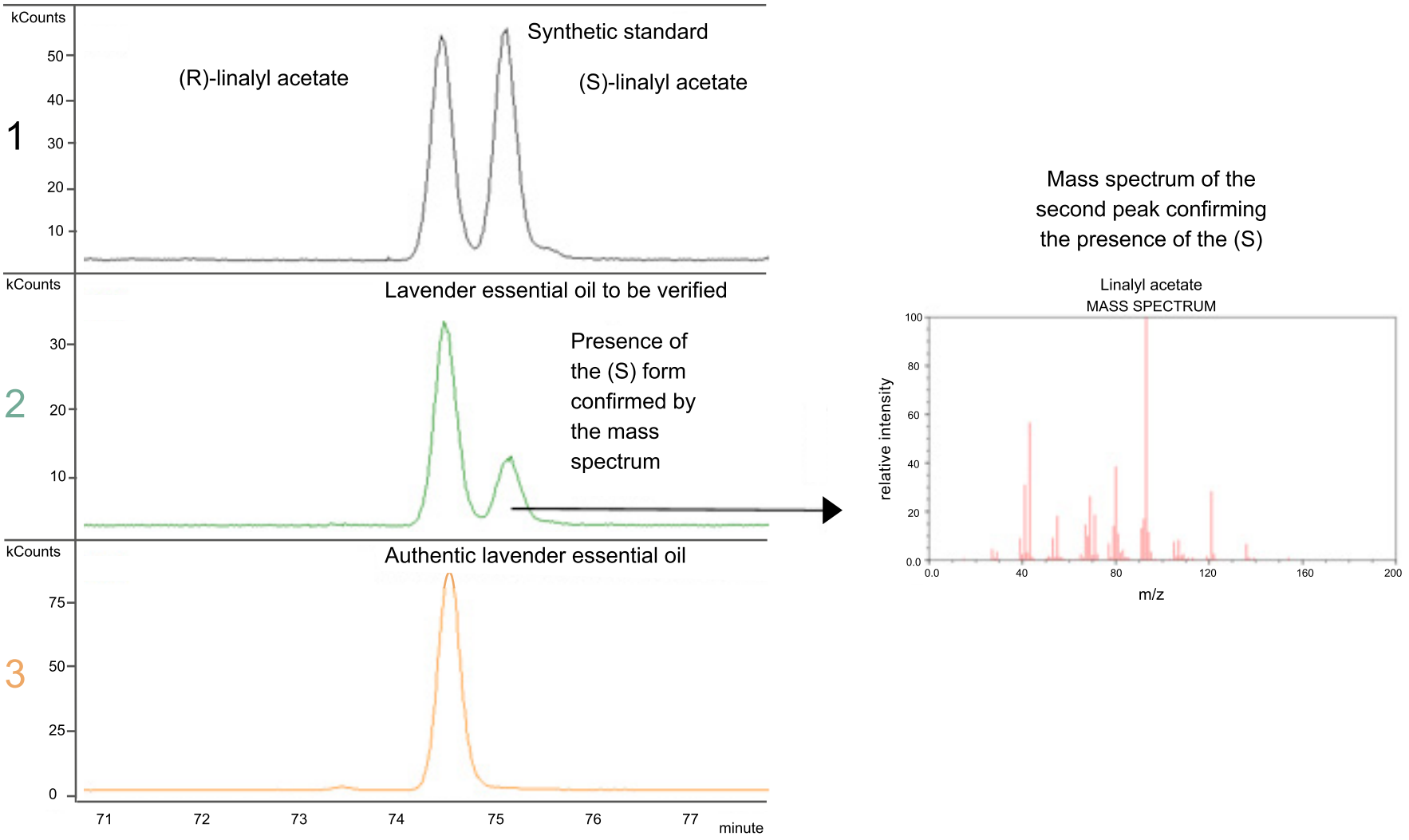


Figure 5. Separation of the (R) and (S) enantiomers of linalyl acetate by gas chromatography in conjunction with mass spectrometry: synthetic molecule (curve 1), lavender essential oil to be tested (curve 2), authentic lavender essential oil (curve 3)

Depending on the nature of the samples to be analysed (formulated flavouring, essential oil, food, etc.), it will often be necessary to make a **sample preparation or extraction** for the purpose of transforming the sample into a form compatible with the analytical technique, and in some instances to enrich the compounds to be analysed to the point where they are detectable.

› In a straightforward analysis (essential oil, target compounds present in sufficient quantity, etc.), this stage will consist of dissolution in a suitable solvent, such as dichloromethane.

› In more complex analyses (high-fat foods, target compounds present only in trace amounts, etc.), the extraction process will be more elaborate and may, for example, include high vacuum distillation.

Frequently, extraction methods of the type used for non-chiral analysis of flavouring compounds in a given matrix will also be suitable prior to chiral analysis. Two-dimensional gas chromatography, combining an achiral column in the first dimension and a chiral column in the second, will considerably increase analytical selectivity, thereby determining an enantiomeric ratio even in the most unfavourable circumstances (analysis of traces in the presence of numerous interferents).

To be valid, a chiral analysis method must demonstrate selectivity (the ability to distinguish between the enantiomers of the marker compound(s)), **specificity** (the absence of interference from other compounds present) and **the absence of artefact formation** (notably by racemisation during sample preparation, extraction or chromatographic separation). This validation can be achieved by analysing authentic samples and/or enantiopure reference compounds and is specific to each compound analysed.

2.3. Enantiomeric ratio stability

In nature, flavouring substances generally take the form of mixtures of their enantiomers. In natural materials, one enantiomer generally predominates.

For example: d-carvone in caraway, l-carvone in spearmint.

However, there are also examples of natural racemic mixtures.

For example: racemic tartaric acid in grapes, citronellal in lemon eucalyptus and gamma-octalactone in raspberries.

Here is a practical example: The great majority of linalool is found in its (R)-(-)- form in cold-extracted bergamot essential oil, basil essential oil and true lavender essential oil, whereas in sweet orange essential oil and strawberry (raw material RM), the (S)-(+)- enantiomer predominates, and in fruits (RM) such as apricot, pineapple, blackcurrant or raspberry, a mixture more or less similar to the racemate is observed.

Subjecting bergamot or true lavender to hydrodistillation, which involves heating at high temperature for varying lengths of time, results in the formation of significant quantities of (S)-(+)-linalool. Furthermore, the presence of an acidic medium such as citrus juice can promote the racemisation of linalool. On the other hand, other chiral compounds are more difficult to racemise; for example, the configuration of beta-Pinene is not affected by heat treatment or the presence of a weak acid (fruit juices, pH of around 3, etc.).

2.4. Building a database

Determining enantiomeric excesses (or enantiomeric ratios) is part of the arsenal of techniques available to analysts required to give an opinion on the naturalness of (chiral) flavouring molecules. However, enantiomeric excess measurement is relevant only when the analyst has access to a reference database.

Where no such internal database exists, either because the laboratory is only just embarking on this activity or because the molecule has not previously been monitored, analysts have no option but to refer to scientific studies published in the literature.

For isolated molecules, a simple determination of rotatory power can be used to check whether the molecule has the expected optical activity (a racemic molecule will remain inactive under polarised light). If the molecule is present in a flavouring or extract, the only solution is often enantioselective chromatography; in most cases, this will involve gas chromatography (GC), and sometimes liquid chromatography (LC) or even supercritical fluid chromatography (SFC).

International scientific literature is full of chirality analysis data for a very wide range of naturally occurring chiral molecules. The analyst is therefore faced with an abundance of information that is not always easy to sort through. Referring to authors with a proven track record in the field is often a way of ensuring that the 'external data' sourced can be used as a reliable reference for the analyses to be conducted internally.

Annex 1 contains a list of authors to which the reader may wish to refer in their possible bibliographic research. It follows that it is very much in the interest of a laboratory to build its own database containing reliable data sourced from literature and, primarily, its own analyses accumulated over the course of its analytical activities.

To ensure that this internal database is maintained and used appropriately by the analyst, we recommend that each result is rigorously recorded, together with all necessary traceability data.

Each laboratory will have its own procedure, but it is customary for the accumulated results of chiral analysis of a molecule in an ingredient, matrix or flavouring to be used to set an internal laboratory standard by expressing the mean and standard deviation of the values measured. This mean value can be used to define a tolerance interval of around twice the standard deviation. Any value measured outside this tolerance interval should be seen by the analyst as a warning sign.

3. Chirality and regulatory compliance

At European level, Regulation (EC) 1334/2008 sets out the regulatory requirements for flavourings and certain food ingredients with flavouring properties.

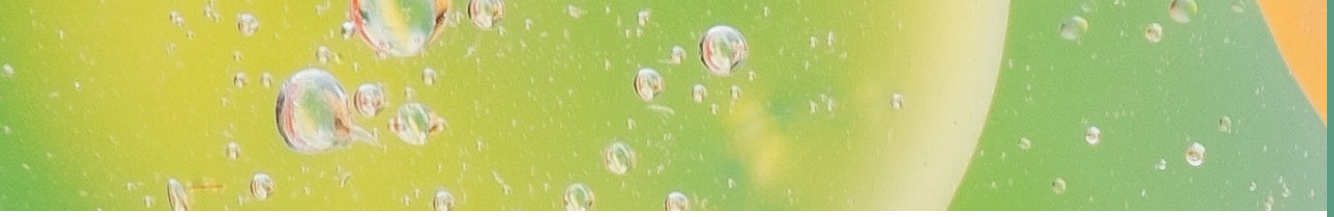
Regulatory compliance of natural status flavourings is governed by multiple factors, as detailed in the document entitled 'Analytical controls and regulatory compliance of natural flavourings' prepared by P3A.
Determining the enantiomeric ratio of substances present in flavouring source materials is one way of obtaining indications of regulatory compliance.

Chiral analysis can raise doubts and identify inconsistencies in flavouring descriptions, particularly where the enantiomeric ratios differ from those known or observed in nature.

That said, chiral analysis alone is not enough to reach a positive or negative conclusion regarding the naturalness of a flavouring. This analysis should be combined with:

› Other molecule identification techniques (isotope analysis, Carbon-14 analysis, detection of the presence of adulterants undetectable by gas chromatography, synthetic impurities, etc.)

› An assessment of the guarantees of process, formulation and other compliance offered by the flavouring producer



For 'natural X flavourings', analysis of the proportion originating from the source of X (minimum 95%) should make it possible to recover the enantiomeric distribution of this source. As regards the proportion not originating from the source (maximum 5%), subject to the naturalness of the processes/compounds, any enantiomeric distribution is permissible in regulatory terms. *So the results obtained from a chiral analysis of a natural X flavouring in its entirety may be deemed compliant, even though they may not perfectly reflect the enantiomeric distribution of the source.*

In the case of natural flavourings (as defined in Article 16.6 of the Flavouring Regulation and therefore not making direct reference to a source), regulatory compliance is much more difficult to assess using chiral analysis. In reality, the most useful aspect of chiral analysis is its ability to determine whether the constituents of the flavouring are demonstrably related to the source named.

Annex 1

Non-comprehensive list of reference authors on chiral analysis

A. MOSANDL
P. SCHREIER
H. CASABIANCA
L. MONDELLO
C. BICCHI
L. SCHILIPILLITI
A. KRÜGER

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